Flow Cytometric Test for Beryllium Sensitivity

Tatyana N. Milovanova,[1] Sicco H. Popma,[2] Sindhu Cherian,[3] Jonni S. Moore,[3,4] and Milton D. Rossman[1]*

[1]Pulmonary, Allergy and Critical Care Division, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania
[2]University of Pennsylvania Cancer Center, Clinical Cell and Vaccine Production Facility, Philadelphia, Pennsylvania
[3]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania
[4]Flow Cytometry and Cell Sorting Facility, Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania

Background: Chronic beryllium disease (CBD) is an occupational granulomatous disorder characterized by hypersensitivity to beryllium, mediated by $CD4^+$ T lymphocytes, and predominantly affects the lungs. In this disorder, lymphocyte proliferative responses to beryllium, measured by ^3H thymidine incorporation, are used for diagnosis of CBD, for screening asymptomatic workers or former workers to detect unrecognized disease, and for surveillance as a bioassay to detect abnormal exposures. Problems with test variability and the use of radioactivity have recently led to the search for alternative methods.

Methods: We applied a 5,6-carboxyfluorescein diacetate succinimidyl ester flow cytometric technique for measurement of mitogen- and antigen-induced T-lymphocyte proliferation to a group of beryllium-exposed sensitized individuals and beryllium-unexposed controls.

Results: We detected mitogen and antigen proliferative responses in $CD3^+$, $CD4^+$, and $CD8^+$ subpopulations. Phytohemagglutinin and *Candida* stimulated $CD4^+$ and $CD8^+$ T-cell responses, but beryllium appeared to stimulate only $CD3^+/CD4^+$ responses.

Conclusions: This technique may provide a sensitive, nonradioactive alternative to the traditional proliferation tests that measure beryllium sensitivity. It offers the added specificity of enabling phenotypic description of the responding cell type and may prove to be easier to standardize for clinical use. © 2004 Wiley-Liss, Inc.

Key terms: lymphocyte proliferation; CD3; CD4; CD8; granuloma; hypersensitivity

Chronic beryllium disease (CBD) is a hypersensitivity granulomatous disease that predominantly affects the lungs (1). Measurement of lymphocyte proliferative responses to beryllium using peripheral blood cells or cells obtained by bronchoalveolar lavage is the standard method of documenting beryllium sensitivity (1–3). Detecting beryllium sensitivity has been useful as part of the diagnostic criteria for CBD (4,5), and cross-sectional studies have demonstrated that detecting beryllium sensitivity is useful for the early identification of subclinical and clinical beryllium-induced disease (5,6). Perhaps even more important, detection of beryllium sensitization has been used as a bioassay for the detection of abnormal environmental exposures (6–14).

The standard method for performing the lymphocyte proliferation assay uses tritiated thymidine to measure DNA synthesis (1). This has been termed the *beryllium lymphocyte proliferation test* (BeLPT) (5). The BeLPT is now required by the Department of Energy of the United States as part of the CBD prevention program (15). In the United States, an estimated 800,000 workers were considered to be at risk for developing CBD (16) in 1978. Because of the sensitivity of the BeLPT in identifying excessive exposures, the number of workers at risk for development of CBD in the United States is probably considerably larger, and there may be more than 1 million workers worldwide (Lee Newman, personal communication) who are at risk. Thus, CBD represents a unique use of lymphocyte proliferation testing (blood and lavage) for diagnosis, screening, and surveillance. However, the widespread use of the BeLPT has been criticized because of the variability of the test (9), despite efforts to standardize the method (15,17).

Recently, flow cytometric methods have been developed to measure the proliferation of cells (18–20). By combining a fluorescent proliferation marker such as 5,6-

*Correspondence to: Milton D. Rossman, M.D., University of Pennsylvania Medical Center, 834 West Gates Building, 3400 Spruce Street, Philadelphia, PA 19104.
E-mail: rossmanm@mail.med.upenn.edu
Received 18 August 2003; Accepted 7 April 2004
Published online 14 June 2004 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.b.20015

carboxyfluorescein diacetate succinimidyl ester (CFSE) and monoclonal antibodies, it is possible to phenotype the cells that proliferate and even determine the precursor frequency of proliferating cells (19,21–23). In human cells, this method of measuring lymphocyte proliferation has been applied to blood cells stimulated by mitogen (18), allogeneic (18,24), Candida (24), or tetanus antigens, and glutamic acid decarboxylase (25) for the measurement of immune reactivity related to transplantation, immunosuppression, and autoimmunity.

Because the tritiated thymidine method of determining lymphocyte proliferation is complex and requires radioactive materials and analysis on several days, in the present study, we investigated whether a beryllium-stimulated blood T-cell response could be measured with a CFSE flow cytometric method. We compared the results of our CFSE method with the results of thymidine uptake in workers exposed to beryllium with previous evidence of hypersensitivity and normal unexposed volunteers.

MATERIALS AND METHODS

Population Tested

Beryllium workers (current and former), referred to the Hospital of the University of Pennsylvania for possible CBD, were tested. Individuals were considered to have beryllium hypersensitivity if they had a positive blood BeLPT on at least two occasions or a positive bronchoalveolar lavage (BAL) BeLPT. CBD was diagnosed when there was evidence of beryllium hypersensitivity with granulomas on biopsy and/or radiologic changes consistent with a granulomatous process. Normal unexposed volunteers were used as controls.

Blood BeLPT (^{3}H-Thymidine Incorporation Assay)

Peripheral blood lymphocytes were isolated from heparinized blood under sterile conditions using density centrifugation with lymphocyte separation medium (ICN Biomedicals Inc., Aurora, OH, USA). Blood BeLPT was performed as previously reported (1). The cells were cultured at a concentration of 2.5×10^5 cells/well. Stimulants included 500 μg/ml of phytohemagglutinin (PHA; L-9132, Sigma Chemical Co., St. Louis, MO, USA), 20 μl/ml of *Candida* (M15; Greer Labs, Lenoir, NC, USA), and 100 or 10 μM of beryllium sulfate (Brush Wellman, Cleveland, OH, USA). After culturing the cells in an incubator (5% CO_2 and 37°C) for 3, 5, or 7 days, the cells were pulsed with ^{3}H thymidine (DuPont NEN, Boston, MA, USA) overnight (i.e., 16–24 h) and then harvested on a filter fiber (Wallac, Turku, Finland). The uptake of ^{3}H thymidine was measured as counts per minute (CPM) by using a Wallac 1205 beta plate liquid scintillation system (Wallac Inc., Gaithersburg, MD). Results were expressed as a stimulation index (mean CPM of test wells/mean CPM of control wells). A positive response was defined as a stimulation index score higher than 3.0 for blood. A positive test required that a positive response be recorded on two different days or at two different concentrations of beryllium.

Surface and Intracellular Staining

Cell surface marker staining was performed as described elsewhere (19). The following monoclonal antibodies were used: phycoerythrin (PE) conjugated to anti-human CD4, tricolor conjugated to anti-human CD3, and allophycocyanin (APC) conjugated to anti-human CD8 (Caltag, Burlingame, CA, USA). The vital dye TO-PRO-3 (Molecular Probes, Eugene, OR, USA) was used to discriminate between live and dead cells (19).

For intracellular staining, cells were fixed for 20 min with 2% formaldehyde at room temperature. After two washes in 0.5% bovine serum albumin (BSA), cells were then permeabilized with 0.1% saponin (Sigma Chemical Co.) in phosphate buffered saline containing 0.5% BSA for 10 min at room temperature. Intracellular staining was performed for 30 min with cells resuspended in a small volume of 0.1% saponin in 0.5% BSA at room temperature. After two washes in a 0.1% saponin, the cells were resuspended in phosphate buffered saline with 0.5% BSA and analyzed by flow cytometry.

CFSE Labeling and Measurement of Proliferation

The CFSE labeling method was adapted from a previously described protocol (19). Aliquots of the same peripheral blood lymphocyte sample used in the BeLPT assay were split and washed three times in 10 ml of phosphate buffered saline (Gibco BRL, Grand Island, NY, USA) at room temperature. The cells were adjusted to 10^6 cells/ml, and CFSE (Molecular Probes) was added at a final concentration of 1 μM/ml. The cells were vortexed for 10 s and then incubated for 10 min in the dark at room temperature with gentle shaking. After incubation, an equal volume of sterile, heat-inactivated human serum (Gibco BRL) was added to the sample for 1 min to quench the reaction. The cells were washed twice and adjusted to a concentration of 2×10^6/ml in 10% heat-inactivated human serum.

After CFSE labeling, the cells were cultured in 24-well plates at 2×10^6 cells/well in the presence of medium alone, PHA, *Candida,* or 10 or 100 μM of beryllium sulfate. The cells were incubated for 7 days at 37°C and 5% CO_2. Preliminary experiments titrated the optimal dose of CFSE and time of incubation (data not shown). Surface labeling was performed at the time of harvest by using CD3TC, CD4PE, and CD8APC in some experiments. TO-PRO-3 was added to define live cells in some experiments.

Flow Cytometric Data Acquisition and Analysis

All data were acquired on a four-color, dual laser (FACSCalibur, Becton Dickinson, San Jose, CA, USA). CFSE was measured in the FL1 channel (530/30-nm bandpass filter), CD4PE in the FL2 channel (585/42-nm bandpass filter), CD3TC in the FL3 channel (670-nm longpass filter) using excitation from the 488 nm blue laser, and TO-PRO-3 or CD8APC in the FL4 channel (661/16-nm bandpass filter) by excitation using the 635-nm red diode laser. Compensation for CFSE in multiparameter flow cytometry is dose dependent and determined empirically.

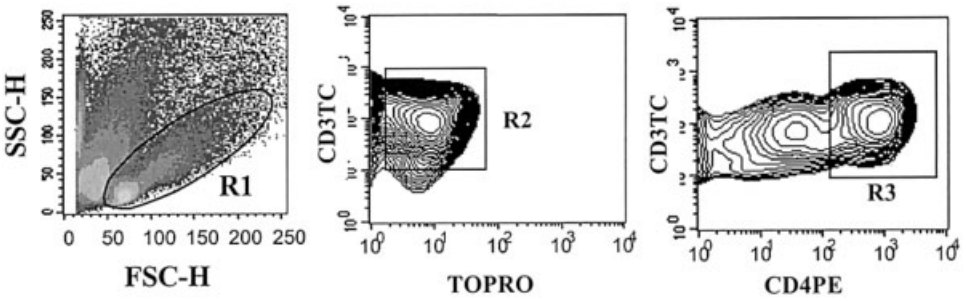

FIG. 1 Gating strategy for beryllium-sensitivity analysis. Regions were set by using PHA-stimulated lymphocytes. For $CD3^+CD4^+$ analyses, an initial region was defined as lymphocytes by light scatter (R1) and then identification of $CD3^+$/TO-PRO-3^- population (R2). $CD3^+CD4^+$ populations were identified as R3. Population-specific proliferation was determined with single-parameter histograms of the identified subsets.

The basic analysis gating strategy was as follows (Fig. 1). For analysis of $CD3^+$ T cells alone, an initial region was defined as lymphocytes by light scatter, and a broad region was set to include medium to high forward/low side scatter events after stimulation with PHA (Fig 1, R1) to include all proliferating lymphocytes. For $CD3^+$ T cells, the R1 data were plotted in a contour plot of CD3TC versus TO-PRO-3 to identify live $CD3^+$ T cells (Fig. 1, R2). The R2 population was expressed on a contour plot of $CD3^+CD4^+$, where a double positive region was defined as R3 (Fig. 1). For final analyses, each population was then analyzed for CFSE (FL1) fluorescence, representing proliferation, on a single-parameter histogram using a logical gate of R1*R2 for $CD3^+$ cells only and R1*R2*R3 for $CD3^+$/$CD4^+$cells. In experiments in which data from only $CD3^+$ or $CD3^+/CD4^+$ cells were collected, a live gate on TO-PRO-3^- ("viable") cells was also used in acquisition. When specific $CD4^+$ and $CD8^+$ T-cell determinations were performed, the viability dye was eliminated (data not shown). The strategy was similar to that with light scatter gating (R1) followed by identification of the $CD3^+$ events using a plot of side scatter versus $CD3^+$ (R2) and $CD4^+$ (R3) and $CD8^+$ (R4) identification followed by single-parameter histogram analysis of the CFSE fluorescence (for $CD4^+$, R1*R2*R3; for CD8, R1*R2*R4*).

Quantitative analysis of proliferation using CFSE has been described previously (19). CFSE flow cytometric data files were analyzed with CellQuest acquisition and analysis software (Becton Dickinson). Fifty thousand events were collected. Data are reported as the proliferative ratio, defined as the ratio of the percentage of divided cells to undivided cells on day 7 (%M2/%M1). The metric of percentage of dividing cells has been used in assays comparing flow cytometry measures of proliferation with standard ^{3}H thymidine methods (18).

Statistical Analysis

All values are presented as mean ± standard error of the mean. Data are presented only if a minimum of 1,000 cells were detected within a population. For the tritiated thymidine test, the results were expressed as a stimulation index, and group differences (between controls and beryllium-sensitive populations) were evaluated with Student's *t* test. For the CFSE tests, because the results were expressed as a proliferative ratio, the Mann-Whitney version of the Wilcoxon signed rank test was used to determine whether there was a difference between the normal donors and the beryllium-exposed patients. Statistical significance was defined as $P < 0.05$.

RESULTS

Beryllium Sensitivity Measured by Thymidine Incorporation

Nine normal unexposed controls and 24 beryllium-exposed individuals previously shown to be sensitized to beryllium were analyzed in the first series of experiments. Four of the 24 were diagnosed with CBD based on the positive blood and previous BAL BeLPT results and granulomas present on biopsy; seven of 24 had a diagnosis of beryllium alveolitis, based on findings of positive blood and BAL BeLPT results without granulomas on biopsy; and 13 had a diagnosis of beryllium hypersensitivity based on positive blood BeLPT results on at least two occasions with negative findings on bronchoscopy. Positive responses were observed in the control and beryllium-sensitized populations for the response to PHA on day 3 and *Candida* on days 5 and 7 (Table 1). There were no significant differences in these responses between these

Table 1
*Mitogen-and Antigen-Induced Proliferative Responses of Cells From Control and Beryllium-Sensitized Subjects Measured by Thymidine Incorporation**

Stimulants	Normal controls (n = 9)	Be-sensitized subjects (n = 24)
Day 3		
Unstimulated (CPM)	571 ± 72.5[a]	598 ± 77.5
PHA (SI)	137 ± 38.9[b]	178 ± 23.2
Day 5		
Unstimulated (CPM)	827 ± 215	988 ± 254
Candida (SI)	13.5 ± 3.3	30.3 ± 9.14
Be 10 μM (SI)	0.72 ± 0.08	12.1 ± 3.96[†]
Be 100 μM (SI)	0.74 ± 0.09	12.8 ± 4.93[†]
Day 7		
Unstimulated (CPM)	1,750 ± 596	2,710 ± 872
Candida (SI)	24.3 ± 8.1	29.2 ± 8.07
Be 10 μM (SI)	0.44 ± 0.08	12.5 ± 4.43[†]
Be 100 μM (SI)	0.52 ± 0.12	11.5 ± 4.86[†]

*Be, beryllium; CPM, counts/minute; PHA, phytohemagglutinin; SI, stimulation index.
[a]Mean ± standard error of the mean of CPM.
[b]Mean ± standard error of the mean of SI.
[†]$P < 0.05$ versus controls.

two groups. In contrast, whereas positive responses to beryllium were not noted in the control group, positive responses were noted to beryllium in the beryllium-sensitized population ($P < 0.05$).

CFSE-Measured $CD3^+/CD4^+$ T-Cell Response to Beryllium In Vitro

To verify that we could detect cell proliferation by flow cytometry, CFSE-labeled peripheral blood mononuclear cells were cultured for 7 days in the presence of culture medium alone, PHA, *Candida*, or 10 or 100 μM beryllium sulfate. After 7 days of culture, cells were harvested and surface stained with CD3TC/CD4PE/TO-PRO-3. PHA and *Candida* were used as positive controls because of their strong T-cell stimulation effect. For negative controls, unstimulated cells labeled with CFSE were used. A live gate was used (TO-PRO-3) to exclude any dead cells that might have lost CFSE.

In the initial series of experiments, we gated on the live $CD3^+$ cell population (TO-PRO-3^-) that were $CD4^+$ high (positive) and calculated the proportion of cells that had divided (proliferative ratio) from the histograms. Flow cytometric analysis of CFSE-labeled lymphocytes showed a typical pattern of proliferating cells detectable in the mitogen- and beryllium-treated cultures, demonstrating that the response to beryllium could be detected. Control cultures (unstimulated) exhibited no loss of CFSE intensity (Fig. 2, Table 2).

Similar to the results with tritiated thymidine, positive responses to PHA and *Candida* were noted for the $CD3^+$ and $CD3^+CD4^+$ cells from the control and beryllium-sensitive populations (Table 2). There was minimal response detected in the $CD3^+$ or $CD3^+/CD4^+$ high cells from controls to 10 or 100 μM $BeSO_4$. In contrast, the cells from the beryllium-sensitive population showed a significant positive response to 10 μM $BeSO_4$ in the $CD3^+$ population and to 10 and 100 μM $BeSO_4$ in the $CD3^+/CD4^+$ population ($P < 0.05$). As expected, no significant differences were noted between the cells from normal subjects and the cells from the subjects with beryllium sensitivity in the CFSE-determined response to PHA or *Candida*. When the values from beryllium-sensitized patients were compared with those obtained from normal controls, it was determined that the CFSE method could discriminate between these two groups. Of note, in some experiments, there appeared to be a positive beryllium response in the $CD3^+/CD4^+$ low population (data not shown).

CFSE-Measured Proliferative Response of $CD4^+$ and $CD8^+$ T Cells to Beryllium In Vitro

In the initial experiments, it was not possible to determine the exact phenotype of the $CD3^+$/CD4 low cells, although in many cases they may have represented $CD8^+$ T cells. Finding a beryllium response in a population of possible $CD8^+$ T cells was unexpected because a $CD8^+$ response to beryllium in vitro was believed to be nonexistent (26). However, in cultured T cells, $CD3^+$ high/$CD4^+$ low T cells may contain not only $CD8^+$ T cells but also $CD4^+$T cells with surface CD4 that has been down regulated. Because we observed in the first series of experiments that gating on live cells, using TO-PRO-3 , did not offer significant advantages over light scatter gating for viabilility, we replaced TO-PRO-3 with anti-$CD8^+$ APC. By using this four-color flow cytometric staining technique, we could measure the proliferation of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cell populations.

$CD3^+$, $CD3^+/CD4^+$, and $CD3^+/CD8^+$ T cells from control (n = 6) and beryllium-sensitized (n = 14) populations responded to PHA and *Candida* in vitro as expected (data not shown). In contrast, whereas a significant difference in the response of the $CD3^+$ and $CD3^+/CD4^+$ T cells from beryllium-sensitized subjects was detected to both doses of $BeSO_4$, no differences were noted in the response of $CD8^+$ T cells from normal donors or beryllium-sensitized individuals, which is consistent with previous reports (Table 3).

To further confirm that the "$CD4^+$ low" response was due to $CD4^+$ cells that may have lost surface expression of this marker, intracellular staining with anti-CD4 was performed to confirm the identity of the cells. T cells from a beryllium-sensitive individual were labeled with CFSE and cultured for 7 days in the presence of 10 or 100 μM beryllium sulfate. The cells were harvested; half of the cells were surface stained with anti-CD4PE/CD8APC, and the remaining cells were permeabilized before staining. In the representative example shown in Figure 3, we identified two populations of $CD4^+$ cells by gating on $CD3^+$ T cells. The $CD4^+$ high population was 27.8% and the $CD4^+$ low population was 37.3% of the T-cell population. However, with intracellular staining, a more homogeneous population of $CD4^+/CD8^-$ cells was observed (58.3%). This suggests that the $CD4^+$ low population was the result of downregulation of surface $CD4^+$ of a proliferated $CD4^+$ population. Surface and intracellular staining results of anti $CD8^+$ were identical at 7.6%.

DISCUSSION

In this study, we applied the CFSE flow cytometric technique to measure sensitivity to beryllium. This method combines immunophenotyping with a measure of mitogen- and antigen-induced lymphocyte proliferations in the same test. There are multiple advantages in replacing a radiochemical assay such as tritiated thymidine incorporation BeLPT with a flow cytometrically based assay. Information is obtained only on viable cells, and proliferation is measured on specific lymphocyte subsets by using fluorescent rather than radioactive reagents. In addition, because the CFSE test records the total number of cell divisions over the entire culture period rather than just the number that is actively synthesizing DNA during a specific period, the cells can be harvested at a single time point. The flow cytometric analysis is straightforward and can be done on commercial clinical flow cytometers and, as shown in this study, can discriminate between normal donors and beryllium-exposed individuals.

CFSE allowed us to determine the ratio of the cells that had divided to undivided cells by day 7 in response to

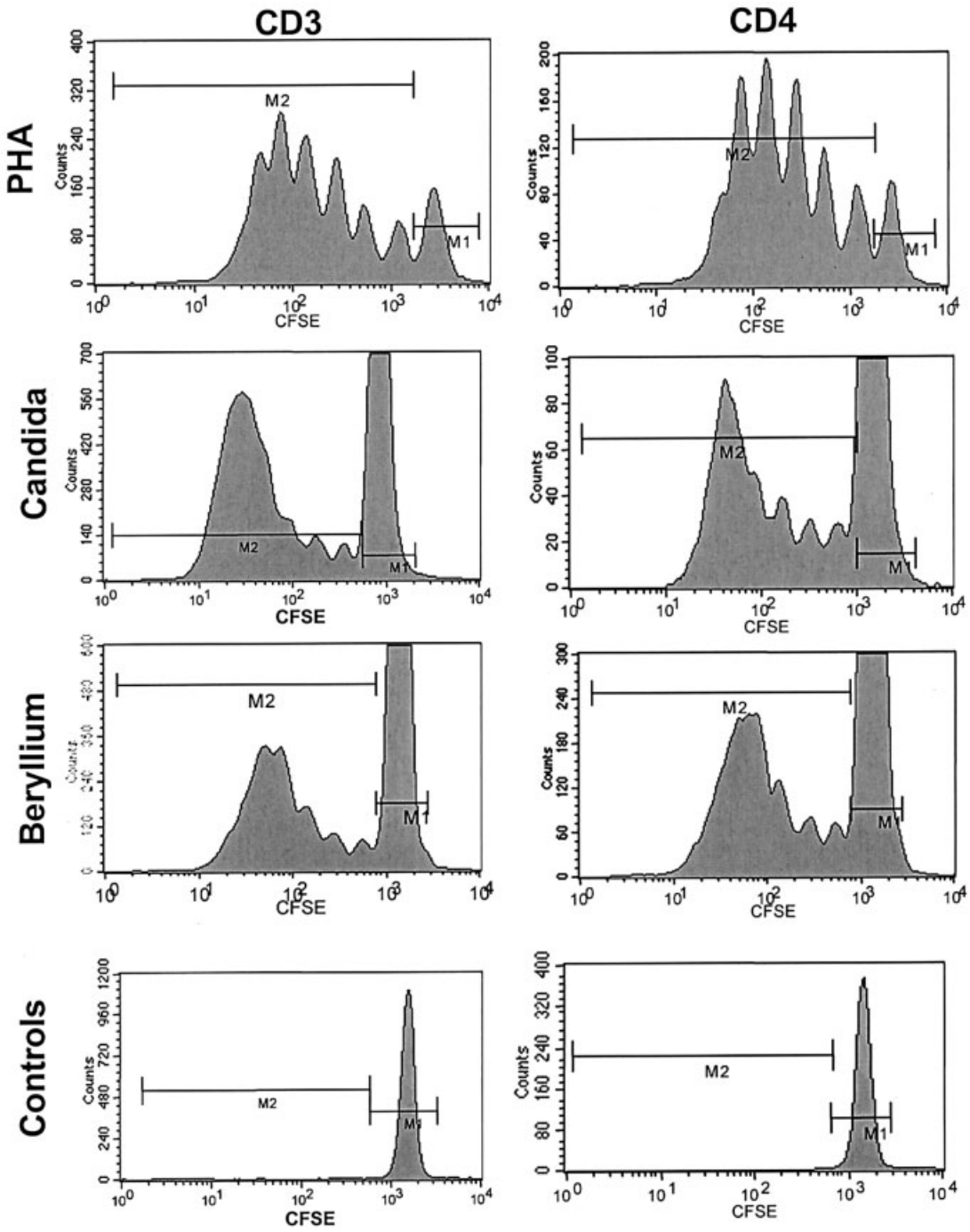

FIG. 2 Example of CFSE-labeled $CD3^+$, $CD4^+$ T-cell proliferation cultured for 7 days with PHA, *Candida*, 100 μM $BeSO_4$, or control. The subject was BeLPT positive. CFSE fluorescence intensity is displayed on a log scale. The undivided cells are indicated by the M1 bar and the divided cell populations by the M2 bar.

Candida or beryllium and to phenotype the responding cells. In our studies, we were clearly able to show that PHA and *Candida* stimulated $CD4^+$ and $CD8^+$ T cells, whereas beryllium appeared to stimulate only $CD4^+$ T cells. If the specific subpopulation of T cells that responds to an antigen is important, then a CFSE-based proliferation assay has a distinct advantage over tritiated thymidine-based assays. Failure to identify the phenotype of the responding cell may have been due to the fact that no correlation was noted between the *Candida*-induced proliferative response and the presence of mucus candidiasis in patients with the human immunodeficiency virus (27).

Table 2
*Mitogen and Antigen $CD3^+$ and $CD3^+/CD4^+$ T-Lymphocyte Responses From Beryllium-Sensitized Subjects and Beryllium-Unexposed Donor Controls Measured by CFSE Dye Dilution**

Subset/treatments	Normal controls (n = 9)	Be-sensitized subjects (n = 24)
Stimulants	$CD3^+$	$CD3^+$
Unstimulated	0.023 ± 0.005[a]	0.037 ± 0.004
PHA	9.853 ± 2.908	10.358 ± 4.730
Candida	0.105 ± 0.041	0.396 ± 0.254
Be 10 μM	0.027 ± 0.005	0.115 ± 0.046[†]
Be 100 μM	0.030 ± 0.008	0.099 ± 0.026
Stimulants	$CD3^+/CD4^+$	$CD3^+/CD4^+$
Unstimulated	0.023 ± 0.005	0.036 ± 0.005
PHA	10.064 ± 2.753	15.756 ± 5.094
Candida	0.076 ± 0.035	0.114 ± 0.020
Be 10 μM	0.027 ± 0.005	0.105 ± 0.038[†]
Be 100 μM	0.029 ± 0.010	0.089 ± 0.027[†]

*Be, beryllium; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; PHA, phytohemagglutinin.
[a]Mean ± standard error of the mean of proliferative ratio.
[†]$P < 0.05$ versus controls.

In contrast to mitogen and allogeneic responses, antigen-specific responses of human peripheral blood are difficult to measure because of the small number of responding cells Antigen-specific responses of lymphocytes can be measured by a variety of methods. Delayed hypersensitivity skin testing is a classic example (28). Cell cycling responses can be measured by radioactive (1) or fluorescent (20) markers of DNA synthesis or by direct cell counts. Cytokine secretion or synthesis is another measure used to detect antigen-specific responses (28).

The BeLPT is a tritiated thymidine-based assay of lymphocyte proliferation to beryllium. The BeLPT is probably the only lymphocyte proliferation test that is routinely used diagnostically. This assay is part of medical screening and surveillance programs to detect beryllium sensitivity in workers occupationally exposed to beryllium (15). The BeLPT has been used in cross-sectional studies and was instrumental in identifying unrecognized cases of CBD and workplace environments where excessive exposure to beryllium was occurring. However, the application of this test to wider populations and routine use has been criticized because of reports of variability in results between laboratories performing the test (8,9,29). Improved tests of beryllium sensitivity might correct this variability.

In the past 20 years, a great deal has been learned about the immunologic response to beryllium. We now know that $CD4^+$ memory T cells accumulate in the lungs (1,26) or skin (14) in response to beryllium. These T cells specifically recognize an unknown antigen and undergo clonal expansion in the presence of beryllium (26,30,31). This antigen is presented to $CD4^+$ T cells by antigen-presenting cells, probably macrophages and dendritic cells, in the context of class II human leukocyte antigen (HLA) (26). In particular, HLA-DP molecules on the surface of the antigen-presenting cells appear to be most important for presenting the beryllium antigen to the α/β-antigen receptor of the T cell (32–35).

$CD4^+$ T cells were thought to mediate the hypersensitivity to beryllium, not only because of their accumulation at the site of the disease activity (1,26) but also because of their ability to respond to beryllium in vitro (26,33,34). This concept was supported by the observation that the proliferative response of lymphocytes to beryllium could be inhibited by antibodies to HLA class II molecules (26,30).

In addition to the ability of beryllium to cause a $CD4^+$ T-cell hypersensitivity response, beryllium may act as an adjuvant and nonspecifically stimulate the innate immune system (36). The mechanism of beryllium's adjuvant activity is unknown, but the recent demonstration of nonspecific stimulation of tumor necrosis factor α release by macrophages (37) suggests that beryllium may nonspecifically interact with macrophages and/or dendritic cells. If nonspecific cytokine release causes cellular proliferation in vitro, then this may be responsible for some of the variability noted in the BeLPT.

Recent reports have also suggested that $CD8^+$ T cells may rarely respond to beryllium (20). This may represent a true $CD8^+$ T-cell response or a cytokine-driven response secondary to the adjuvant effects of beryllium. Because the $CD4^+$ T cell response in CBD is thought to be the pathogenic T-cell response (30), an assay such as the CFSE-based flow cytometric measurement of lymphocyte proliferation, which can differentiate $CD4^+$ from $CD8^+$ T-cell responses, may be important.

Use of CFSE to assess proliferation offers the ability to use a variety of metrics. In addition to the proliferation ratio presented in this report, additional metrics could include the responder frequency and the relative proliferation rate of responding cells (38,39). Although these data would certainly be of interest from a theoretical perspective, the practical value of this data in defining beryllium hypersensitivity remains to be determined.. Additional studies will have to be performed in defined patient populations to determine the particular metric that would provide the most clinical power. In these preliminary

Table 3
*Beryllium Responses of $CD3^+$, $CD3^+/CD4^+$, and $CD3^+/CD8^+$ Subpopulations From Control and Beryllium-Sensitized Subjects as Measured by CFSE Dye Dilution**

Subsets/treatments	Normal controls (n = 6)	Be-sensitized subjects (n = 14)
CD3+		
Be 10 μM	0.012 ± 0.002[a]	0.156 ± 0.114[†]
Be 100 μM	0.013 ± 0.002	0.188 ± 0.137[†]
CD4+		
Be 10 μM	0.015 ± 0.003	0.164 ± 0.112[†]
Be 100 μM	0.015 ± 0.002	0.210 ± 0.114[†]
CD8+	(n = 4)	(n = 11)
Be 10 μM	0.013 ± 0.003	0.016 ± 0.003
Be 100 μM	0.011 ± 0.004	0.019 ± 0.002

*Be, beryllium; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester.
[a]Mean ± standard error of the mean of proliferative ratio.
[†]$P < 0.05$ versus controls.

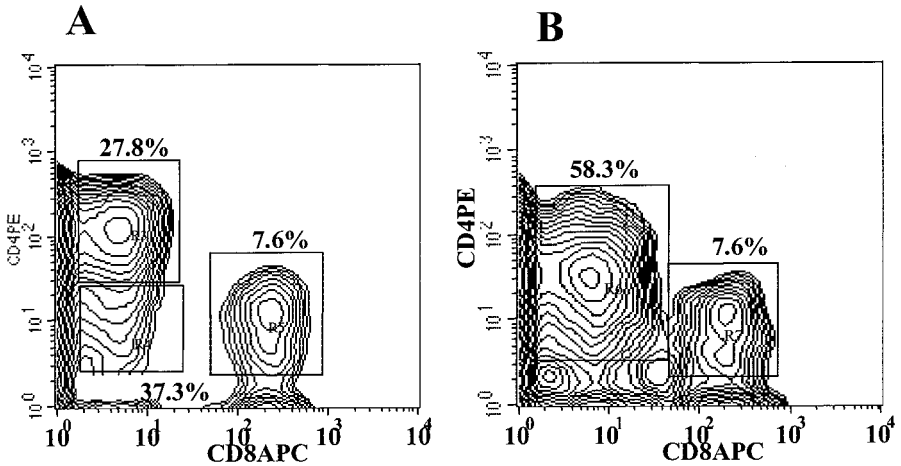

FIG. 3 Contour plots of cells of an individual with a positive BeLPT. Cells were labeled with CFSE and then cultured in the presence of 100 μM $BeSO_4$ for 7 days. Cells were harvested and divided into two aliquots. **A:** One aliquot was surface stained with CD3, CD4, and CD8. **B:** Another aliquot was permeabilized (intracellular) before staining with CD3, CD4, and CD8. Small cells were initially identified by forward and side scatter and then gated on $CD3^+$ cells. The contour plots illustrate the CD4 and CD8 staining patterns of the $CD3^+$ population. The percentages represent the proportion of the cells in the selected population in reference to the initial small cell population identified.

studies, the simple calculation of the proliferation ratio, similar to BeLPT results, may identify a population with beryllium hypersensitivity.

In conclusion, we have demonstrated that multiparameter flow cytometry with fluorescent tracking dyes may be useful for enumerating and describing antigen-specific proliferating cells that represent a small proportion of the original population. The CFSE flow cytometric method described in this study does not use radioactivity and can identify a specific cell population, and one time point summarizes the proliferation response over the entire culture period. This type of assay may be particularly important when knowing the proliferative response of a particular cell phenotype is important (e.g., $CD4^+$ T cells in patients with the human immunodeficiency virus or CBD) and thus offers a clinically useful alternative to traditional radioactive lymphocyte proliferation tests.

ACKNOWLEDGMENTS

The authors thank J. Liang , M. Yuan and Andrew Bantly for technical support and Mary McNichol for help in preparation of this report.

LITERATURE CITED

1. Rossman MD, Kern JA, Elias JA, Cullen MR, Epstein PE, Preuss OP, Markham TN, Daniele RP. Proliferative response of bronchoalveolar lymphocytes to beryllium. A test for chronic beryllium disease. Ann Intern Med 1988;108:687–693.
2. Kreiss K, Newman LS, Mroz MM, Campbell P A. Screening blood test identifies subclinical beryllium disease. J Occup Med 1989;31:603–608.
3. Newman LS, Kreiss K, King TE Jr, Seay S, Campbell PA. Pathologic and immunologic alterations in early stages of beryllium disease. Re-examination of disease definition and natural history. Am Rev Respir Dis 1989;139:1479–1486.
4. Rossman MD. Chronic beryllium disease: diagnosis and management. Environ Health Perspect 1996;104S:945–947.
5. Kreiss K, Miller F, Newman LS, Ojo-Amaize EA, Rossman MD, Saltini C. Chronic beryllium disease—from the workplace to cellular immunology, molecular immunogenetics, and back. Clin Immunol Immunopathol 1994;71:123–129.
6. Kreiss K, Wasserman S, Mroz MM, Newman LS. Beryllium disease screening in the ceramics industry. Blood lymphocyte test performance and exposure-disease relations. J Occup Med 1993;35:267–274.
7. Henneberger PK, Cumro D, Deubner DD, Kent MS, McCawley M, Kreiss K. Beryllium sensitization and disease among long-term and short-term workers in a beryllium ceramics plant. Int Arch Occup Environ Health 2001;74:167–176.
8. Deubner D, Kelsh M, Shum M, Maier L, Kent M, Lau E. Beryllium sensitization, chronic beryllium disease, and exposures at a beryllium mining and extraction facility. Appl Occup Environ Hyg 2001;16:579–592.
9. Deubner DC, Goodman M, Iannuzzi J. Variability, predictive value, and uses of the beryllium blood lymphocyte proliferation test (BLPT): preliminary analysis of the ongoing workforce survey. Appl Occup Environ Hyg 2001;16:521–526.
10. Deubner DC, Lowney YW, Paustenbach DJ, Warmerdam J. Contribution of incidental exposure pathways to total beryllium exposures. Appl Occup Environ Hyg 2001;16:568–578.
11. Kolanz ME. Introduction to beryllium: uses, regulatory history, and disease. Appl Occup EnvironHyg 2001;16:559–567.
12. Kolanz ME, Madl AK, Kelsh MA, Kent MS, Kalmes RM, Paustenbach DJ. A comparison and critique of historical and current exposure assessment methods for beryllium: implications for evaluating risk of chronic beryllium disease. Appl Occup Environ Hyg 2001;16:593–614.
13. Kreiss K, Mroz MM, Zhen B, Wiedemann H, Barna B. Risks of beryllium disease related to work processes at a metal, alloy, and oxide production plant. Occup Environ Med 1997;54:605–612.
14. Fontenot AP, Maier LA, Canavera SJ, Hendry-Hofer TB, Boguniewicz M, Barker EA, Newman LS, Kotzin BL. Beryllium skin patch testing to analyze T cell stimulation and granulomatous inflammation in the lung. J Immuno1 2002;168:3627–3634.
15. Chronic beryllium disease prevention program. Office of Environment, Safety and Health, Department of Energy. Final rule. Fed Regist 1999;64:68854–68914.
16. National occupational hazard survey. DHEW Publication 78-114. National Institute for Occupational Safety and Health; 1978. Rockville, MD.
17. Frome EL, Newman LS, Cragle DL, Colyer SP, Wambach PF. Identification of an abnormal beryllium lymphocyte proliferation test. Toxicology 2003;183:39–56.
18. Popma SH, Krasinskas AM, McLean AD, Szeto WY, Kreisel D, Moore JS, Rosengard BR. Immune monitoring in xenotransplantation: the multiparameter flow cytometric mixed lymphocyte culture assay. Cytometry 2000;42:277–283.
19. Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. J Clin Invest 1997;100:3173–3183.
20. Farris GM, Newman LS, Frome EL, Shou Y, Barker E, Habbersett RC, Maier L, Smith HN, Marrone BL. Detection of beryllium sensitivity using a flow cytometric lymphocyte proliferation test: the Immuno-Be-LPT. Toxicology 2000;143:125–140.
21. Givan AL, Fisher JL, Waugh M, EmstoffMS, Wallace PK. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. J Immunol Methods 1999;230:99–112.
22. Wells AD, Walsh MC, Sankaran D, Turka LA. T cell effector function

and anergy avoidance are quantitatively linked to cell division. J Immunol 2000;165:2432–2443.
23. Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods 2000;243:147–154.
24. Angulo R, Fulcher DA. Measurement of *Candida*-specific blastogenesis: comparison of carboxyfluorescein succinimidyl ester labelling of T cells, thymidine incorporation, and CD69 expression. Cytometry 1998;34:143–151.
25. Mannering SI, Morris JS, Jensen KP, Purcell A W, Honeyman MC, van Endert PM, Harrison LC. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. J Immunol Methods 2003;283:173–183.
26. Saltini C, Winestock K, Kirby M, Pinkston P, Crystal RG. Maintenance of alveolitis in patients with chronic beryllium disease by beryllium-specific helper T cells. N Engl J Med 1989;320:1103–1109.
27. Leigh JE, Barousse M, Swoboda RK, Myers T, Hager S, WolfNA, Cutright JL, Thompson J, Sobel JD, Fidel PLJ. *Candida*-specific systemic cell-mediated immune reactivities in human immunodeficiency virus-positive persons with mucosal candidiasis. J Infect Dis 2001; 183:277–285.
28. Mazurek GH, LoBue PA, Daley CL, Bernardo J, Lardizabal AA, Bishai WR, Iademarco MF, Rothel JS. Comparison of a whole-blood interferon *y* assay with tuberculin skin testing for detecting latent *Mycobacterium tuberculosis* infection. JAMA 2001;286:1740–1747.
29. Newman LS, Mroz MM, Maier LA, Daniloff EM, Balkissoon R. Efficacy of serial medical surveillance for chronic beryllium disease in a beryllium machining plant. J Occup Environ Med 2001;43:231–237.
30. Fontenot AP, Falta MT, Freed BM, Newman LS, Kotzin BL. Identification of pathogenic T cells inpatients with beryllium-induced lung disease. J Immunol 1999;163:1019–1026.
31. Fontenot AP, Kotzin BL, Comment CE, Newman LS. Expansions of T-cell subsets expressing particular T-cell receptor variable regions in chronic beryllium disease. Am J Respir Cell Mol BioI 1998;18:581–589.
32. Richeldi L, Sorrentino R, Saltini C. HLA-DPB1 glutamate 69: a genetic marker of beryllium disease. Science 1993;262:242–244.
33. Lombardi G, Germain C, Uren J, Fiorillo MT, du Bois RM, Jones-Williams W, Saltini C, Sorrentino R, Lechler R. HLA-DP allele-specific T cell responses to beryllium account for DP-associated susceptibility to chronic beryllium disease. J Immunol 2001;166:3549–3555.
34. Fontenot AP, Torres M, Marshall WH, Newman LS, Kotzin BL. Beryllium presentation to CD4+ T cells underlies disease-susceptibility HLA-DP alleles in chronic beryllium disease. Proc Natl Acad Sci USA 2000;97:12717–12722.
35. Rossman MD, Stubbs J, Lee CW, Argyris E, Magira E, Monos D. Human leukocyte antigen class II amino acid epitopes: susceptibility and progression markers for beryllium hypersensitivity. Am J Respir Crit Care Med 2002;165:788–794.
36. Lee JY, Atochina 0, King B, Taylor L, Elloso M, Scott P, Rossman MD. Beryllium, an adjuvant that promotes gamma interferon production. Infect Immun 2000;68:4032–4039.
37. Amicosante M, Berretta F, Franchi A, Rogliani P, Dotti C, Losi M, Dweik R, Saltini C. HLA-DP-unrestricted TNF-alpha release in beryllium-stimulated peripheral blood mononuclear cells. Eur Respir J 2002; 20: 1174–1178.
38. Lyons AB. Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. Immunol Cell Biol 1999;77: 509–515.
39. Bernard S, Pujo-Menjouet L, Mackey MC. Analysis of cell kinetics using a cell division marker: mathematical modeling of experimental data. Biophys J 2003;84:3414–3424.